TOWARDS A BETTER UNDERSTANDING OF EUROPEAN OYSTER *OSTREA EDULIS* BREEDING: RESULTS OF THE 2003-2004 ACRDP PROJECT – BEDFORD INSTITUTE OF OCEANOGRAPHY

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ABSTRACT

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The European oyster (*Ostrea edulis*) was introduced to the Nova Scotia aquaculture industry 30 years ago. The stocks were imported from naturalized populations in Maine, whose ancestors originated from the Netherlands. In past years, Nova Scotian hatcheries have successfully produced *Ostrea edulis* spat, but in 2001 and 2002 the two remaining hatcheries in the province suffered 100% larval mortality. One of the factors that may have contributed to the collapse is a suspected loss of genetic diversity due to the limited number of individuals used to establish the Maritimes stocks, and the inevitable subsequent inbreeding during propagation of these populations. Using microsatellites, we found that some genetic erosion has occurred in the Maritime populations, with the largest loss of alleles found in the hatchery stocks. In spite of this loss, genetic diversity and heterozygosity in the Maritimes populations are still relatively high.

While the impact of inbreeding and heterosis on performance (survival, growth, settlement success) could not be evaluated in this project due to mortalities, we found no evidence of selfing (self-fertilization) but did find evidence that mass spawning is dominated by a few individuals.

The impact of grading spat was also evaluated. Smaller oysters (normally discarded as "runts") showed increased growth rate (% mass and size) when competition from larger oysters was removed and when density was lower.

Recommendations for broodstock management of *Ostrea edulis* and the direction of future studies of this species are summarised at the end of this report.

RÉSUMÉ

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L'huître européenne (*Ostrea edulis*) a été introduite pour l'industrie aquacole de Nouvelle-Écosse il y a 30 ans à partir de stocks importés de populations naturelles du Maine dont les ancêtres provenaient des Pays-Bas. Les écloseries néo-écossaises ont dans le passé produit du naissain d'*Ostrea edulis* avec succès, mais en 2001 et 2002, les deux écloseries existantes dans la province ont connu 100% de mortalités larvaires. Un des facteurs qui ont pu contribuer à ces échecs est la suspicion d'une perte de variabilité génétique due au nombre limité d'individus utilisés pour l'établissement des stocks des Maritimes et l'augmentation de la consanguinité inévitable lors de la propagation de ces populations. En utilisant des microsatellites, nous avons trouvé qu'il y a eu de l'érosion génétique dans les populations des Maritimes, avec la plus grande perte d'allèles dans les stocks d'écloseries. Malgré cette érosion, la diversité génétique et l'hétérozygosité des populations des Maritimes sont encore relativement élevées. Bien que l'impact de la consanguinité et de l'hétérosis sur la performance (survie, croissance, succès de fixation) n'a pas pu être évalué dans ce projet en raison des mortalités, nous n'avons pas trouvé d'évidence d'auto-fertilisation mais des éléments probants indiquant que les pontes en masse sont dominées par quelques huîtres. L'impact du triage du naissain a aussi été évalué. Les huîtres les plus petites (normalement rejetées car chétives) ont montré un taux de croissance (% masse et taille) accru lorsque que la compétition de la part des huîtres plus grandes était éliminée et lorsque la densité d'élevage était plus faible.
Des recommandations pour la gestion des géniteurs d'*Ostrea edulis* ainsi que des avenues de recherche sont résumées à la fin de ce rapport.

INTRODUCTION

In the mid-eighties, a tremendous potential for the development of the European (also called Belon or flat) oyster in Nova Scotia was identified (Newkirk et al., 1995; Enright, 1995). Nonetheless, unlike other cultured bivalves in this region, this non-native species must be propagated in hatcheries and spat supply was recognised as a major constraint. Sixteen years later, after some successes and failures, the industry is still facing challenges in this area: in fact, since 2001, larvae and spat have experienced 100% mortalities in the two commercial hatcheries in Nova Scotia. Water quality, such as variation in temperature or organic / bacterial load, along with genetic erosion were suspected as the cause of the mortalities.

The preservation and utilisation of the genetic variability already present in Nova Scotia European oyster stocks is a critical and complex issue. We currently have a finite resource of European oyster genetic diversity within the province of Nova Scotia. Improper genetic management will quickly lead to a loss of this variability and a deterioration of broodstock quality. This could potentially jeopardise the future of this species in Nova Scotia. The benefits of selective breeding as a means of enhancing production has been well demonstrated and the principle is routinely applied in aquaculture. In shellfish, there have been numerous studies demonstrating the value of selective breeding under laboratory conditions, but there has been only limited use of these approaches under commercial conditions (Newkirk, 1988).

A considerable amount of information needs to be gathered in order to develop a coherent breeding program for the European oyster. This is one of the most difficult shellfish species to be brought under genetic control since individually controlled matings, an important element of a breeding program, are particularly difficult to manage (*e.g.* no influence on the timing of fertilisation). To complicate matters further, hermaphroditic individuals with the ability to switch sex within the same spawning season have been observed. Whether this factor translates into a high amount of "selfing" or self-fertilisation within a group is unknown.

A new project, building upon the results of 2002/2003 ACRDP project # MG-01-06-013 "Development of a broodstock genetic program for the European oyster *(Ostrea edulis)*" (Vercaemer et al., 2003), was initiated in 2003 with two objectives:

1. Use the existing genetic variability of various sources of European oysters previously identified by microsatellite technology, to identify potentially useful strains, and evaluate potential strain differences in performance, and
2. Design a selective breeding program suitable for the European oyster as a means of improving performance and minimising inbreeding levels

To achieve objective 2., we needed to:

- Understand European oysters individual reproductive success in both hatchery mass spawning systems and in the field;
- Estimate the impact of selection on genetic diversity;
- Estimate the impact of inbreeding and heterosis on settlement rate, post-settlement growth and survival of European oysters.

Thus, to design an appropriate broodstock genetic program for the European oyster, we need to develop a method of exerting stricter control during mating and evaluate the extent and impact that inbreeding and heterosis have on subsequent performance. In the future, we will need to evaluate the heritability of commercially important traits.

1. EVALUATING GENETIC VARIABILITY IN N.S. BROODSTOCK OYSTERS (CONTINUED FROM 2002/2003)

The advent of DNA fingerprinting, especially the development of microsatellites, has facilitated investigations into the mechanics of breeding, including in aquaculture research (O'Reilly and Wright, 1995; Herbinger *et al.*, 2003). Unique DNA banding patterns that are inherited in a Mendelian fashion are used to recreate the pedigree of individuals. This information is then used to carry out a wide range of genetic analyses as well as to design mating schemes. The 2002/2003ACRDP project allowed us to assess the level of genetic variability in different strains of European oysters from Nova Scotia (Lunenburg, Port Medway, Cape Sable, "wild" Sambro) and New Brunswick (Lockhart Lake) and compare them with samples of naturalized populations from Maine, British Columbia and Europe (Vercaemer *et al.*, 2003). This was done using 5 microsatellite loci developed by IFREMER (Launey, 1998, Launey *et al.*, 2002). After optimizing the microsatellite assays, collecting oyster tissue samples from various groups, DNA was extracted from oyster tissue samples and all samples were genotyped at the 5 loci. Genetic analyses were performed using genetic software. In particular, we compared the amount of genetic diversity (as measured by allelic richness) present in the Canadian populations to a set of weakly differentiated natural populations from Northern Europe (Launey, 1998) and from Maine. At each locus, some genetic erosion can be seen with smaller numbers of alleles being observed in the Canadian collections even though sample numbers were higher. Depending on the locus, some Canadian populations lost between 3 and 8 alleles when compared with the Maine populations. Thus, it would appear that **there is some degree of on-going genetic erosion in the artificially propagated populations.**

We also looked at the amount of genetic diversity present in each of the Canadian populations. Allelic richness, corrected for unequal sample size clearly indicates that the Lockhard Lake and Sambro populations are the most genetically diverse. This is probably due to the fact that the oyster population in Lockhard Lake is a relatively large naturalised population and that the population in Sambro constitutes a small naturalized reservoir of the original transfer from Maine. The 4 remaining populations appeared similar, with slightly higher diversity in the Pacific Coast population. Each of the 5 loci still reveals fairly high allelic richness and it would appear that, as of 2002, **there is still a reasonable level of genetic diversity in the Canadian collections overall.** This appears to be the case despite the fact that these populations have been isolated from their ancestral European, and subsequently, Maine populations for several generations, and were propagated in hatcheries.

In 2003, additional oysters were sampled and genotyped from 2 re-naturalized populations – more individuals from Sambro, N.S. and individuals from a new site: Blind Bay, N.S (25 km, south of Halifax, NS) were added to the database. No oysters were found at the Argyle or Ship Harbor locations but there are anecdotal reports that a few European oysters may be located on the Eastern shore of Nova Scotia.

Three additional microsatellite loci were tested, of which one *(Oedu* HA7) was optimized (Table 1) and added to the list of the 5 loci used in 2002/2003. Only the new individuals collected in 2003 were genotyped at this locus.

Table 1. Characteristics of microsatellite loci in *Ostrea edulis*. Size range is in base pairs and T_a is the annealing temperature.

Locus	Repeat	Size range	T_a (°C)	Reference
*Oedu*T5	(CA)	106-174	56	Launey *et al.*, 2002
*Oedu*H15	(ATCT)	175-227	50	*Idem*
*Oedu*J12	(GT)	217-265	50	*Idem*
*Oedu*O9	(GA)	145-183	50	*Idem*
*Oedu*U2	(AC)(AG)	158-214	60	*Idem*
OeduHA7	(GA)	161-207	56	Sobolewska *et al.*, 2001

The genotypic data for the new individuals were added to our database (see Appendix A), and 2002/2003 results were re-analyzed to incorporate the new individuals (allelic richness, relatedness, etc.). Allelic richness has been corrected for differences in sample size using the CONTRIB software (Petit, 1999). Results are shown in Table 2.

Table 2. Sample size (n) and total number of alleles (– *corrected number for sample size*) per locus for each population. NS hatcheries, Maritimes naturalized, and Maine naturalized combined for comparison.

Population	n[1]	Number of alleles					
		*Oedu*U2	*Oedu*T5	*Oedu*H15	*Oedu*O9	*Oedu*J12	Average
PAC	30	16 – *16.00*	12 – *11.93*	8 – *8.00*	9 – *9.00*	11 – *11.00*	11.2 – *11.20*
LUN	68	14 – *12.62*	12 – *11.39*	9 – *7.86*	9 – *8.16*	13 – *11.59*	11.4 – *10.32*
MED	39	15 – *13.95*	13 – *11.44*	8 – *7.89*	9 – *8.26*	9 – *8.02*	10.8 – *9.91*
CAS	125	18 – *14.42*	17 – *11.85*	7 – *6.87*	10 – *8.37*	16 – *8.92*	13.6 – *10.09*
Total NS hatcheries	**232**	**18**	**17**	**9**	**11**	**20**	**13.6**
BLB	36	11 – *10.91*	12 – *12.00*	8 – *7.77*	7 – *6.86*	7 – *6.84*	9.0 – *8.88*
SAM	57	15 – *14.21*	17 – *14.72*	10 – *9.37*	12 – *10.53*	15 – *11.53*	13.8 – *12.07*
LLO	148	24 – *18.44*	18 – *14.63*	11 – *9.24*	11 – *9.05*	18 – *12.70*	16.4 – *12.81*
Total Maritimes naturalized	**241**	**25**	**19**	**12**	**12**	**20**	**17.6**
CUH	100	22 – *20.26*	23 – *16.39*	14 – *11.88*	16 – *12.10*	19 – *15.02*	18.8 – *15.13*
BOH	89	22 – *19.84*	25 – *18.58*	11 – *10.14*	12 – *10.45*	21 – *17.44*	18.2 – *15.29*
BHB	65	21 – *17.42*	16 – *13.89*	9 – *8.71*	12 – *9.98*	15 – *12.16*	14.6 – *12.43*
Total Maine naturalized	**254**	**28**	**27**	**14**	**17**	**22**	**21.6**

PAC: Pacific Coast, LUN: Lunenburg, MED: Port Medway, CAS: Cape Sable, BLB: Blind Bay, SAM: Sambro, LLO: Lake Lockhart, CUH: Cundy Harbor, BOH: Boothbay Harbor, BHB: Blue Hill Bay.

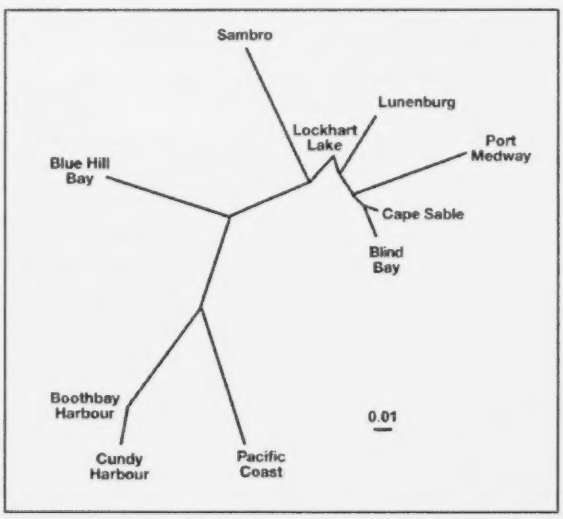
(1) The sample size is given here as an indication only; not all samples amplified at each locus. The PAC collection is the smaller collection; corrected numbers of alleles are equal to the actual number of alleles for all loci but locus T5, where, for the BLB collection, 29 samples only amplified at that particular locus.

The combined Nova Scotia hatchery stocks show the fewest number of alleles compared to the Maritime naturalized populations and the Maine populations as previously described in Vercaemer *et al.* (2003). Allelic richness corrected for unequal sample size clearly indicates that the Blind Bay population is the least diverse in the Canadian collections. This population is not likely a truly "re-naturalized" population as it consists solely of large adult oysters, remnants of a grow-out site used by the Blandford hatchery (SFT Venture, which closed in 1994), without any visible evidence of new "wild" production. This observation of lower diversity in this population is consistent with our previous conclusion that **there is some degree of on-going genetic erosion in the artificially propagated populations** (Vercaemer *et al.*, 2003).

Figure 1 displays the phylogenic tree constructed using Nei's standard genetic distances (Nei, 1978) using the GENETIX (Belkir *et al.*, 1996) and the PHYLIP (Felsenstein, 1993) software packages. The tree compares well with previous results in Vercaemer *et al.*, (2003): the Maritime populations cluster together, and are quite divergent from the Maine populations which cluster together with the Pacific Coast population. The intermediate position of the Sambro oysters may reflect the fact that they are from an older re-naturalized population consisting of Maine oysters that were maintained at Dalhousie University in Halifax for several years before being released. The results also reflect the founding events that led to the establishment of the Pacific Coast population through the transfer of oysters from California, Scotland and Maine, probably via Nova Scotia, over the last 20 years.

The close clustering of the Nova Scotian populations is explained by their recent common ancestry and the exchanges of individuals that have taken place between these populations. For example, the fact that the Lake Lockhart and Lunenburg populations cluster closely together reflects the fact that the Lake Lockhart population was established using mainly oysters from Lunenburg stocks.

Figure 1. Unrooted Neighbor-Joining tree (Saitou and Nei, 1987) obtained from genetic distances.



2. CAPE SABLE 2002 SPAT FAMILY COMPOSITION

This analysis was undertaken in an attempt to understand the individual reproductive success of the European oyster in a mass spawning event and its impact on genetic diversity.

2.1. Background information

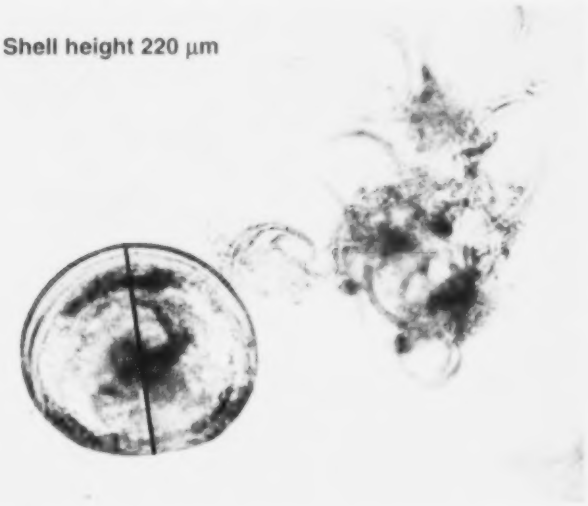
As a spin-off of the 2002/2003 ACRDP project, 35 oysters from **Cape Sable**, NS were genotyped and conditioned for mass-spawning which occurred at the end of June/beginning of July 2002 in the BIO shellfish research hatchery. No other oysters had been conditioned or spawned in the BIO hatchery that year. **These oysters from Cape Sable group were the only ones that produced viable larvae and spat in Nova Scotia in 2002** and below is the sequence of events:

- April 19 - May 26, 2002: preconditioning of 35 oysters from Cape Sable
- May 26 - June 28, 2002: conditioning in 250 L tank
- June 29 -July 3, 2002: releases A, B and C (release B was directly transferred to industry), release A was split into two 250 L tanks raised at different temperatures 15 and 20°C while release C was raised in one 250 L tank at 18°C
- July 4- July 26: releases A and C were raised at densities from to 2 to 5 larvae/mL, regular examinations showed healthy growing larvae (Figure 2)
- July 27- Aug. 2, 2002: releases A and C underwent metamorphosis and 3 successive sets for release A were kept separately.
- Overall, there were 5 groups of spat raised in the shellfish research hatchery at BIO in 2002/2003 as described below:

Temperature for larval rearing/ code	Description of spawning/set events
15°C	Larval release A
18°C	Larval release C
20°C -1	Larval release A – 1st set
20°C -2	Larval release A – 2nd set
20°C -3	Larval release A – 3rd set

In total, 80,000 healthy spat were produced, 50% were kept in the BIO hatchery and 50% were sent to the industry in August, September and October of 2002. Most of the BIO spat were further sent to industry in May 2003.

Figure 2. Picture of a D10 larvae (release A raised at 20°C) with faeces

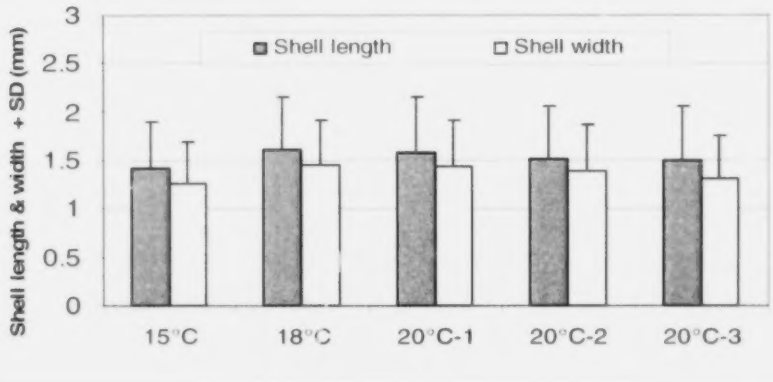


2.2. Sampling, performance and genetic analysis

The spat that remained at BIO (5 groups consisting of ~2,000 spat/group, gropups defined as described above) were maintained in the same conditions *e.g.* temperature and feeding regimes. From each of the 5 groups, 250 spat were randomly sampled in the spring of 2003, at 10 months of age, for length and width measurements (April 28 - May 2, 2003). To assess family composition in groups of juveniles with different performances, the 50 largest and 50 smallest juveniles (see Figure 3) from each group (a total of 500 spat/juvenile) were genotyped at 6 loci.

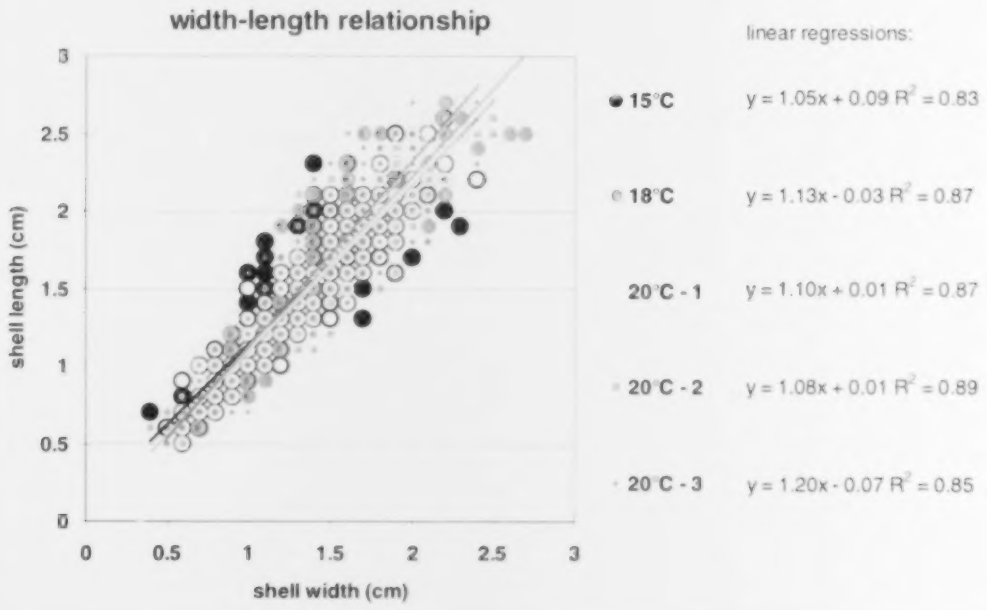
The performance in terms of length and width reached at 10 months of age does not seem to be overall related with the larval rearing temperature (Figure 3).

Figure 3. Shell length and width for the 5 groups of oyster spat (defined as different larval rearing conditions), 250 oysters/group.



Also, there were no observable shape differences between the different temperature groups (Figure 4).

Figure 4. Width-length relationship for the 5 groups of oyster spat (defined as different larval rearing conditions).



The allelic richness (number of alleles per locus) was compared between the 500 Cape Sable spat, their 35 putative parents (mass spawning) and the available reference database of 125 oysters from Cape Sable. Results are presented in Figure 5. The observed number of alleles is lower in the parental group and even lower in their offspring group compared to the Cape Sable reference population. The loss of rare alleles ranged from 1 to 8 alleles for the parents, depending on the locus. The offspring showed an even greater loss (1 to 10 alleles).

Figure 5. Allelic frequencies at 5 loci of a reference database of 125 oysters from Cape Sable, the 35 putative parents of the 2002 mass spawning and 500 spat from that mass spawning. Numbers in brackets indicate number of alleles (allelic richness).

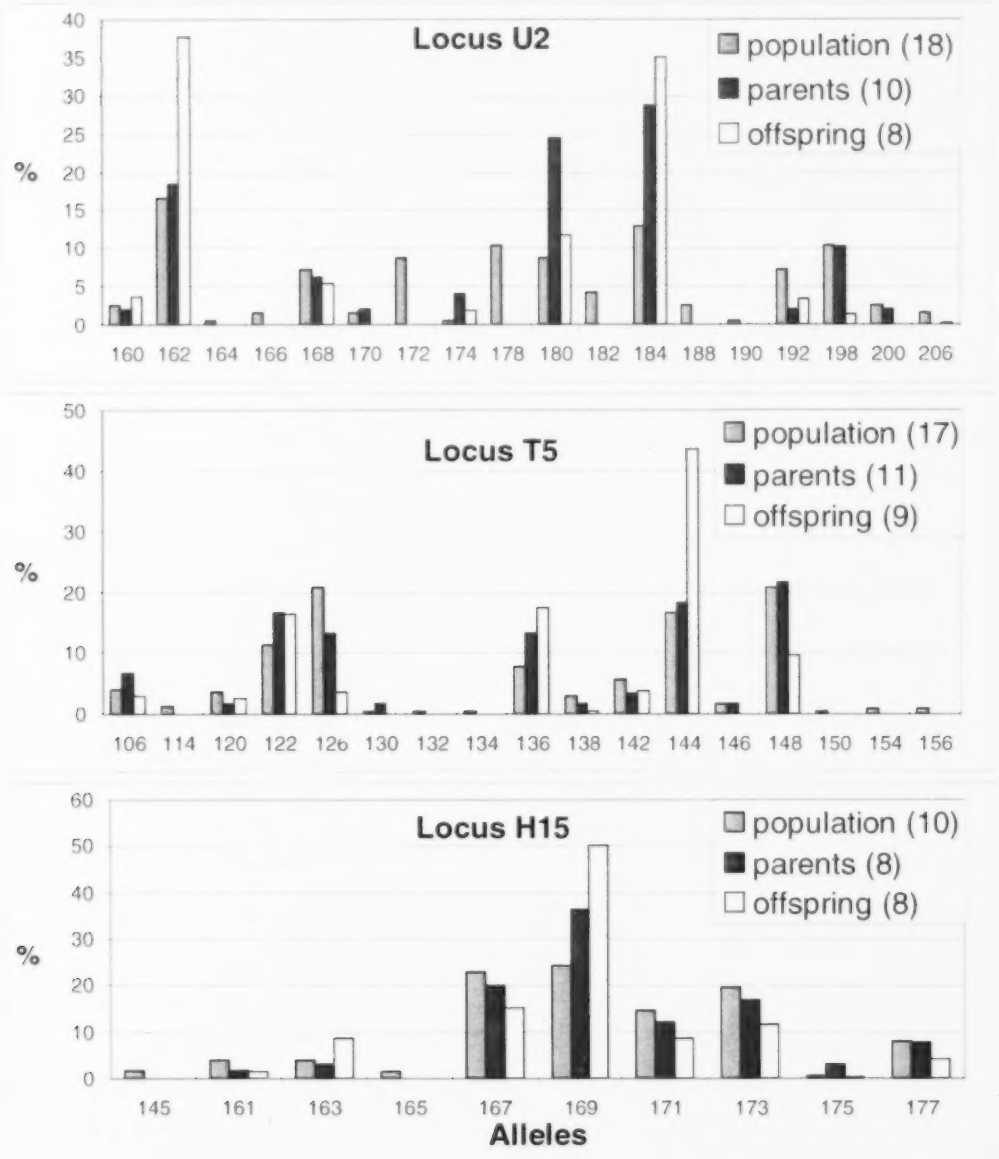
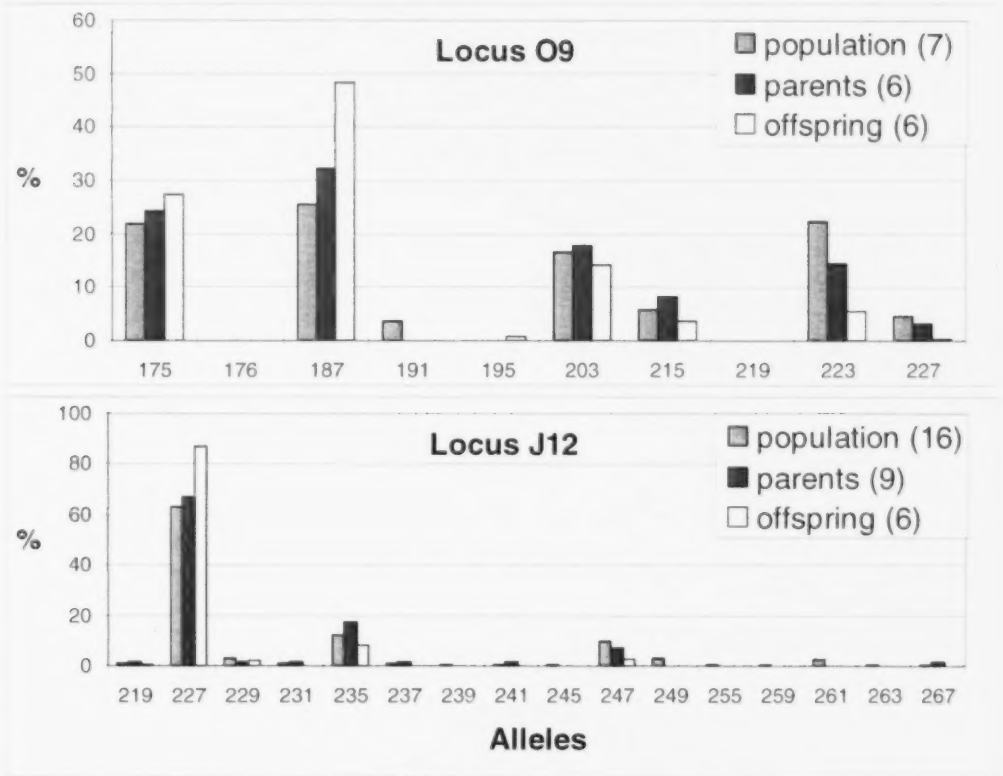


Figure 5. Continued.



Nonetheless, there seems to be no difference in allelic richness between small and large offspring (i.e. largest and smallest spat of all groups combined) for the 6 loci used (Figure 6) nor any differences in heterozygosity related to size (Table 3).

Figure 6. Allelic richness of the largest and smallest Cape Sable 2002 offspring

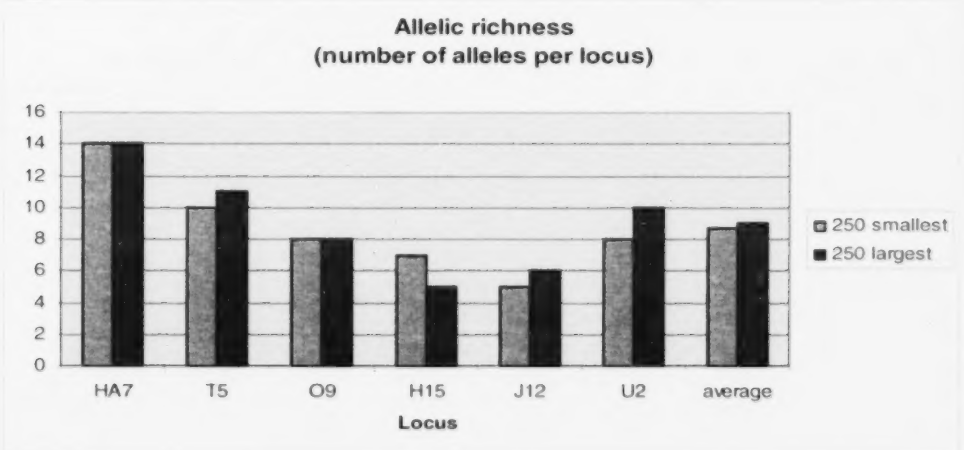


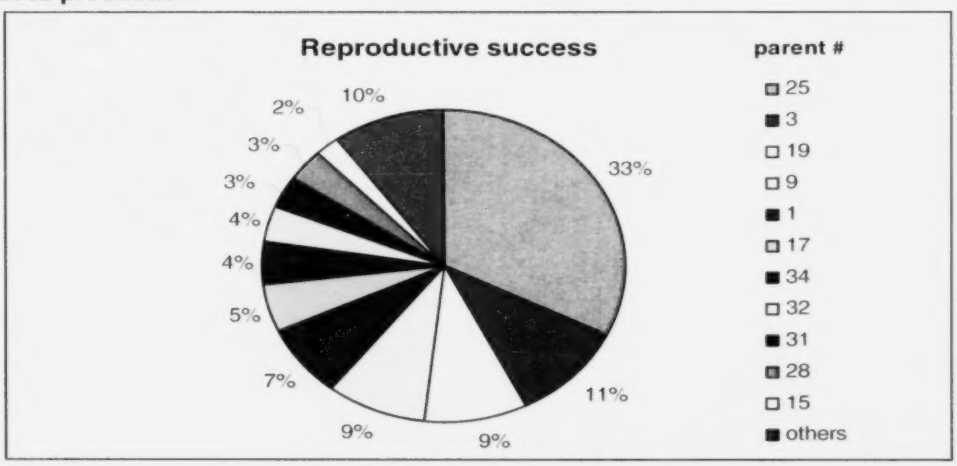
Table 3. Heterozygosity (Hz) of largest and smallest Cape Sable 2002 offspring.

Group	Sample size	Loci typed	Unbiased Hz	Unb. Hz SD	Observed Hz	Obs Hz SD
smallest	250	6	0.6697	0.0850	0.6437	0.0130
largest	250	6	0.6172	0.0798	0.6474	0.0125

The parentage analysis using the software PAPA (Duchesne *et al.*, 2002), showed that the Cape Sable 2002 mass spawning was dominated by a few factorial crosses. Overall, 239 out of 500 offspring could be attributed to a single cross without ambiguity, while the remaining 261 offspring had ambiguous parentage. There was no obvious difference between the small and large offspring or between the various groups in terms of the percentage of unambiguously attributed offspring. The offspring with ambiguous parentage could either not match any parental crosses, probably as a result of a genotyping errors and/or null alleles (shown to exist at least one locus – H15), or on the contrary could match more than one parental cross, probably because of the limiting discriminating power resulting from using a limited number of loci compounded by the presence of missing data (non- amplifying individuals).

One particular problem in interpreting the parental contribution is that the same individual could potentially act as both male and female in different crosses which is what we could be seeing here. Figure 7 represents the proportional parental representation among the 2 x 239 identified parents of the unambiguously assigned offspring. One oyster, #25, (probably a female) was a parent in 1/3 of the cases. The parental oysters #3, #19, #9, #1 and #17, were seen in crosses with putative female #25 and were probably males. They each contributed at least 5% of the offspring. However, about 3.9% of the offspring appeared to have derived from crosses among the parents #3, #19, #9, #1 and #17. This may be an erroneous parentage result, due to genotyping errors, or may be due to hermaphroditism among some of these individuals. Overall, 6 oysters contributed to ~75% of the offspring (and only 11 oysters contributed to 90% of the offspring). The parentage assignment program PROBMAX (Danzmann, 1997) produced similar results.

Figure 7. Reproductive success or contribution of parental oysters to the Cape Sable 2002 production.



Parentage analysis in this study suffered some limitations:

- The genotype of parents must be almost complete (in this case, our database is missing some marker data for 15 parents out of 35 and then only ~50% of the offspring could be attributed to an unambiguous cross).
- The number of loci is the most important limiting factor for parentage analysis and we had only 6, one of which had null alleles, and a couple had a small number of alleles.
- The process is time consuming and complex (presence of full sibs, half sibs, possible hermaphroditism, null alleles, ...).

The contribution of the parental oysters to the two different releases A and C is uneven in most cases (Table 4). Furthermore, the male oysters #3 and #9 significantly contributed more to the production of larger and smaller offspring, respectively. Discarding small offspring in this instance would have resulted in discarding most of a particular cross.

Table 4. Contribution of specific parents (>5%) to the Cape Sable 2002 offspring.

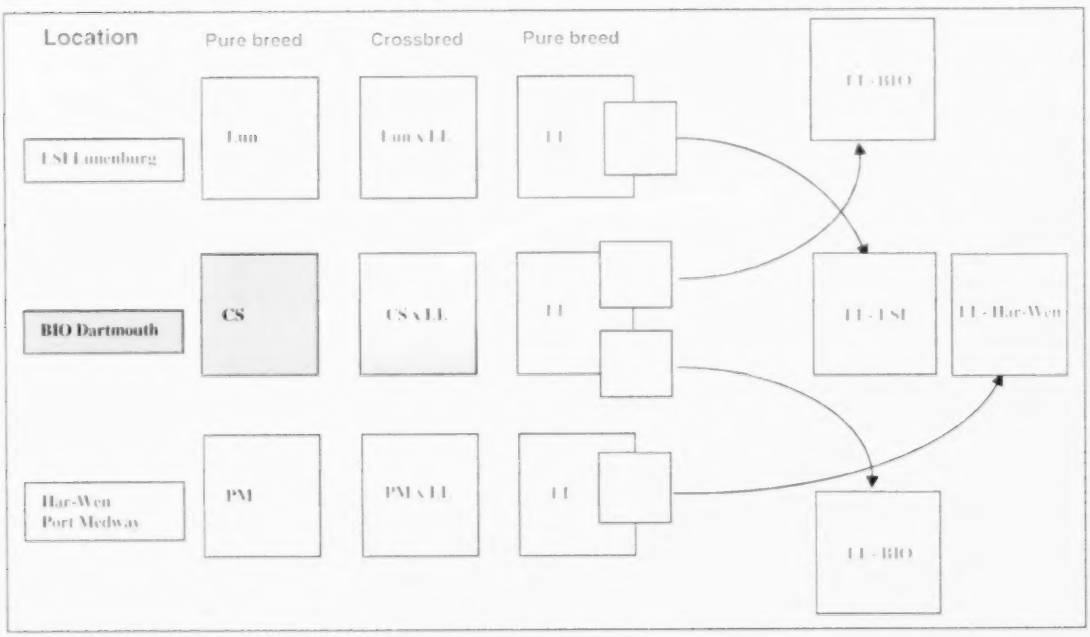
Parent ID #	Number of crosses	% of total offspring production	% contribution to:		% contribution to:	
			All releases A	release C	Large offspring	Small offspring
25	154	32.3	50.5	20.3	57.8	42.2
3	49	10.3	15.3	9.5	81.6	18.4
19	44	9.2	12.7	12.2	59.1	40.9
9	42	8.8	14.2	4.1	28.6	71.4
1	35	7.3	9.8	10.8	48.6	51.4
17	25	5.2	8.7	1.4	48.0	52.0
others	128	26.8				

3. CONTROLLED MASS SPAWNING IN TANKS: ASSESSMENT OF THE IMPACT OF HETEROSIS ON PERFORMANCE WITH 4 CROSS-BRED STOCKS AND CONTROL LINES IN 3 ENVIRONMENTS

3.1. Experimental design

Different stocks may display differences in performance (growth, survival, shape) when subjected to similar conditions. Also, cross-bred populations have been shown to perform better than control lines in *Ostrea edulis* (Newkirk and Haley, 1982; Baud et al., 1997; Naciri-Graven et al., 1999) and other bivalves (Hedgecock et al., 1996), an improvement attributed to heterosis. To test if performance can be improved by heterosis, four distinct groups were simultaneously cross-bred: Lake Lockart (LL), Lunenburg (Lun), Cape Sable (CS) and Port Medway (PM) in the spring of 2003 at LSI - Lunenburg, Har-Wen Farms - Port Medway and BIO – Dartmouth, along with the appropriate control lines (2 pure "strains" per crossbreed). Also, in the context of suspected environmental problems in the commercial hatcheries, larvae from Lake Lockhart, the common group in the 3 hatcheries (LSI, Har-Wen Farms and BIO), were cross–transferred between sites to assess environmental effects (see Figure 8).

Figure 8. Experimental set-up for 2003 mass spawning (Lun: Lunenburg, CS: Cape Sable, PM: Port Medway and LL: Lake Lockhart). The location of the mass spawnings is indicated on the right of the diagram. Cape Sable stock was the only stock from which spat were obtained in 2002.



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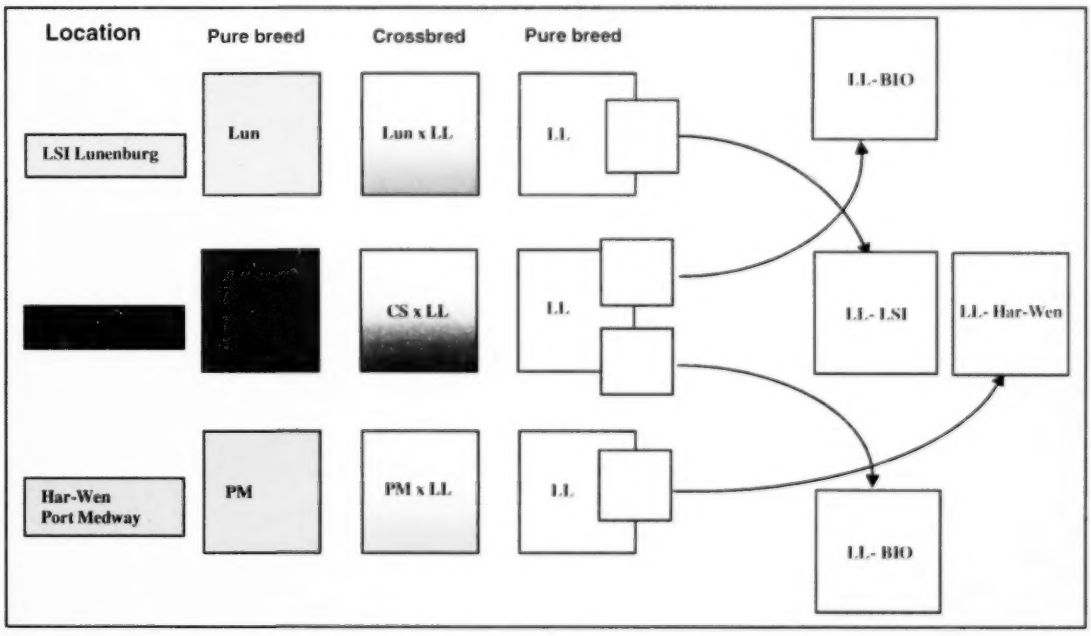


3.2. Mass spawnings at the Shellfish research hatchery at BIO-Dartmouth

On March 9, 2003, 50 LL oysters arrived at BIO and were placed in ambient water at 2°C. On March 21, 53 CS oysters arrived at BIO are were placed in ambient 5°C water. Temperature was slowly raised for both groups to 12 °C by March 28. On April 1, the two groups were moved from their fibreglass holding tanks to three 250L insulated tanks for a 6 week final conditioning period and mass spawning: 30 oysters from Cape Sable (CS tank), 18 oysters from Cape Sable and 15 oysters from Lake Lockhart (CSxLL tank) and 30 oysters from Lake Lockhart (LL tank). Similar set-ups were arranged at LSI and Har-Wen Farms with larger volumes.

At BIO, aborted eggs were found on May 10 and May 14 in the CS tank. Larvae were released from May 20, 2003 to June 7, 2003, in 3 to 5 releases per tank , 0.08 to 8.00 $x10^6$ larvae per release. Table 5 below summarizes the events at the BIO hatchery:

Table 5: Performance of the different stocks, mass spawned and raised at the Shellfish research hatchery at BIO - Dartmouth (LL: Lake Lockhart, CS: Cape Sable)

	Number of Animals	Date of Larval Release	Comments:
LL	30	May 20, May 21am, May 21 pm, May 28, June 7	All releases set at D17-D22 All Dead D51-84
CS x LL	18CS + 15LL	May 15, May 27, June 2	1st release did not set and died on D18, 2nd release did not set and died on D19, 3rd release set D18, and died on D31
CS	30	May 20, May 21, May 27, June 7, (aborted eggs May 10 and May 14)	Set at D22, all died D19-84

3.3. Larvae transferred to and from the Shellfish research hatchery, BIO - Dartmouth

On April 11, 2003, 3 day old larvae (D3) from LL parents spawned in Port Medway were transferred to BIO and, on May 4, new larvae (D0) from LL parents were received at BIO from Lunenburg (Table 6). This part of the experiment was intended to assess the environmental conditions of larval rearing.

Table 6: Performance of the Lake Lockhart stock (LL) raised or transferred to the Shellfish research hatchery at BIO - Dartmouth

LL Stock From:	Date of Larval Release	Date of Set at BIO	Comments
Dartmouth-BIO (LL@BIO)	May 20, May 21	D21-22	Died D68-69
Port Medway (LL@MED)	April 7	D16-D23 (10 scrapes)	400,000 spat alive on D44. On D65 150,000 spat given to industry partners. Remaining died on D115
Lunenburg (LL@LUN)	May 4	D18-19	Died D35

On May 23, 2003, a subset of the LL@BIO group (2 million D3 larvae) was transferred to Lunenburg but not to Port Medway due to lack of space and the potential risk of a horizontal transmission of a suspected infection from the LL@LUN group (see Table 6).

3.4. Results and conclusion

The results of the different spawnings at all locations were disappointing. In both commercial hatcheries, Port Medway and Lunenburg, all larvae and spat from any origin died by July 31, after a sudden change in water temperature (water turned cold very quickly). Similar mortalities were observed, although to a lesser extent, at the BIO shellfish research hatchery. This may be explained by the fact that the intake for the BIO hatchery is much deeper (20 m) than the shallow intake at the two commercial hatcheries. The deeper intake may have buffered the variable environmental conditions better than the shallow, more variable intakes. At the Dartmouth - BIO site, the different releases were raised separately in 20L buckets to 250L tanks according to densities. All larvae groups set with the exception of CS release #5. By August 13, all spat in the different downwellers had died even though standard husbandry protocols (see Appendix B) had been strictly followed. Therefore, the evaluation of performance in terms of survival and growth, settlement success, weight gain and shape and the family composition could not be performed.

4. CONTROLLED MATINGS IN BROOD HOUSING UNITS (BHU) FOR SELFING, HETEROSIS AND PERFORMANCE STUDIES

The final part of the project involved controlled matings in small chambers (Brood Housing Units) to assess "selfing" (hermaphrodism previously observed on histological surveys) and its impact on production and genetic diversity. Controlled matings allow as well the assessment of inbreeding on performance with family lines of closely related matings and non-related matings.

4.1. Background information

Several histological surveys of mature European oysters have consistently revealed a high incidence of hermaphrodism (Mallet, pers. obs.). It is not known whether these individuals are simultaneous hermaphrodites, or if they harbour both types of gametes, which are then released sequentially in the same spawning season. They may have evolved a system similar to plants where genetically similar gametes are incompatible. Nonetheless, "selfing" may potentially be an important means of fertilisation in European oysters and its current impact on seed production and maintenance of genetic diversity is unknown.

Obtaining larvae from selfing and from matings between closely related and non-related oysters could help assess the impact of inbreeding on performance. It is well documented that inbreeding in bivalves tends to reduce performance and increases vulnerability to environmental changes or diseases (Beattie et al., 1987; Ibarra et al., 1995; McGoldrick and Hedgecock, 1997; Bierne et al., 1998; Naciri-Graven et al., 2000). However, successful inbred lines have been developed that are commercially viable (Mallet and Haley, 1983).

4.2. Set-ups and results

To address the impact of selfing and inbreeding on performance, ~120 oysters were conditioned for spawning in the spring of 2003 and at the beginning of 2004. Three attempts were made to spawn groups, pairs and individuals (selfing) in Brood Housing Units designed at BIO (Figure 8). The temperature and feeding regime were similar to conditions in commercial conditioning systems (Newkirk, 1986). The three set-ups were conditioned as follows:

Figure 8. Brood Housing Units (winter 2004, set-up #3) in a flow-through mini habitat system designed at BIO.

Spring 2003

The first BHUs were set up on April 1, 2003 for a 5 week conditioning period. Twelve pair matings of Cape Sable, NS, oysters or Lake Lockhart, NB, were arranged as follows (each cell represents an unit):

Set-up #1

Cape Sable x Cape Sable	Cape Sable x Cape Sable (1)	Cape Sable x Cape Sable (2)
L Lockhart x L Lockhart	Cape Sable x Cape Sable	Cape Sable x Cape Sable
L Lockhart x **L Lockhart**	L Lockhart x L Lockhart (1)	L Lockhart x L Lockhart (2)
L Lockhart x **L Lockhart**	Lake Lockhart x Lake Lockhart	Lake Lockhart x Lake Lockhart

Total: 24 oysters Successful releases in red Mortalities in blue

One pair mating (i.e. one unit) and one individual oyster in three units died (highlighted in blue in the above cells) during the conditioning period. Out of the 8 remaining units, 4 pair matings (highlighted in red in the above cells) released larvae, between May 9 to May 17, 2003:

CS x CS (1) release on May 17, larvae died on D16
CS x CS (2) release on May 11, larvae died on D22
LL x LL (1) release on May 17, set on D20, died on D42
LL x LL (2) release on May 9, spat died on D47

A second BHU system was set up on June 25, 2003 with oysters from two re-naturalized populations (Sambro, NS and Lake Lockhart, NB) for a 5 week conditioning period with 15 units (6 single and 9 pairs) as follows:

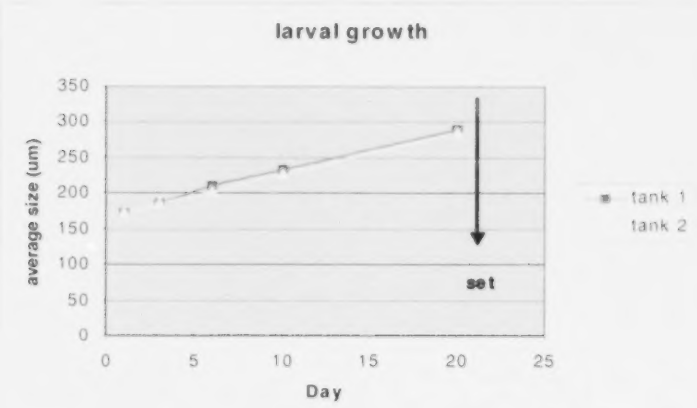
Set-up #2

Sambro	Sambro	Sambro
Lake Lockhart	Lake Lockhart	Lake Lockhart
Sambro x Lake Lockhart	Sambro x Lake Lockhart	Sambro x Lake Lockhart
Sambro x Sambro	Sambro x Sambro	Sambro x Sambro
Sambro x Sambro	Sambro x Sambro	Sambro x Sambro

Total: 24 oysters Successful releases in red No mortality

None of the 6 single individuals produced larvae. One pair out of the nine released larvae on August 5, 2003. This pair produced 3.7x 10^6 larvae (average size of 173 µm). Two 250L tanks were kept at a density of 3 larvae/mL (750,000 larvae). Both larvae cultures looked healthy through the larval phase and set from D22 to D26 (see Figure 9). Oyster spat were raised in five downwellers (see Figure 15 in Appendix B) at a density of 100,000 spat per downweller from D28 to D51 in a 1,000L tank at 19°C. At D51 (September 25, 2003), 250,000 spat were given to each of the two commercial hatcheries (1-3 mm shell height).The spat remaining at BIO were subsequently graded and placed into three downwellers in a 250L tank at a density of 10,000 spat per downweller. They were fed 1.5L of mixed algae on change days (Mondays, Wednesdays, Fridays) and 1.0L on non-change days (~40-60,000 cells/mL).

Figure 9. Larval growth for cross 2003 Sambro x Lake Lockhart



Only the cross Sambro 09 X Lake Lockhart J108 produced larvae. **That cross represents the only viable larvae and spat produced in Nova Scotia in 2003.** Ten larvae from this cross were genotyped at 6 loci to confirm parentage, and proved to be the result of a pair mating and not selfing. Larvae were sampled over time (D2, D6, D14, D20, D28, D48, D53, D93 and D121) to check the resolution of the genotyping at different larval size. A procedure was developed to extract DNA from larvae as small as 180 microns (see Appendix C).

Spat from the above cross were separated into large, medium and small groups, according to size. Duplicated upwellers (2" diameter, see Figure 10) of 50g total biomass for each group were then raised over a 45 day period (December 22, 2003 to February 4, 2004). Changes in mass and length x width for the groups were estimated by measuring oysters in a sample size of 50 to determine if the smaller oysters (normally discarded as "runts") would show increased growth rate when competition from larger oysters were removed. Results are presented in Figure 11. Increases in percentage of mass and size increase were found to be greater for the smaller oyster groups than the larger groups.

The small spat groups (3.9 mm +/-1.4 SD) were then divided into 3 upwellers with different densities (12.5, 25 and 50g/upweller) in duplicate (a and b) on February 4, 2004 and measured again on February 18, 2004. Percentage mass and size increase over this short period of time (15 days) was found to be greater when densities were lower (Figure 12). Both sizing and stocking density experiments were done at 15-18°C temperature and 31‰ salinity; upwellers were fed daily 4-5L/day of mixed MONO/TISO/CHGRA algal cultures (~40-60,000 cells/mL).

Figure 10. Picture of the upwellers in the Shellfish research hatchery, BIO

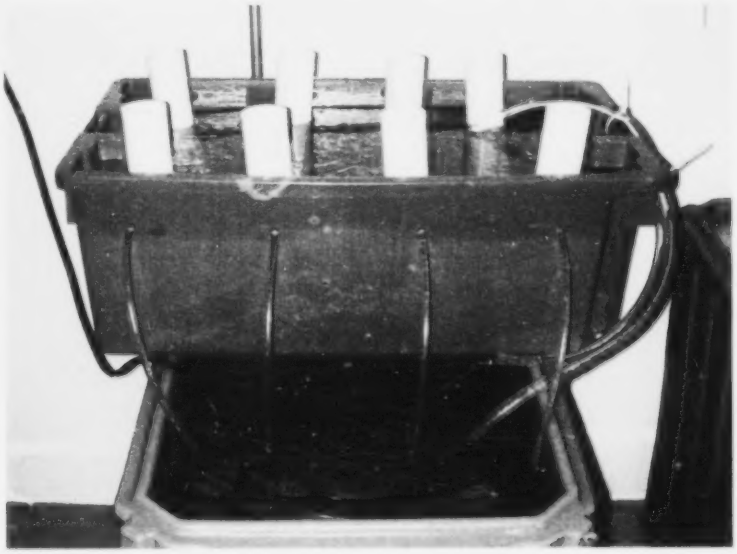
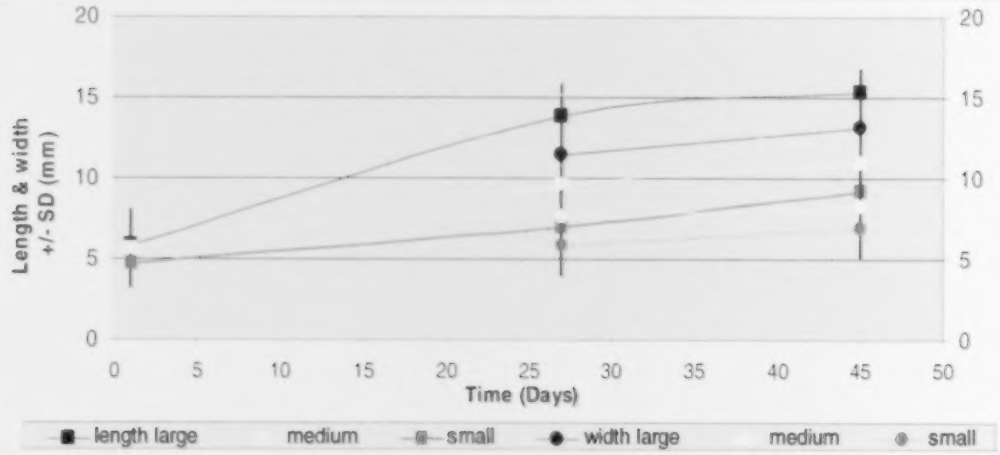
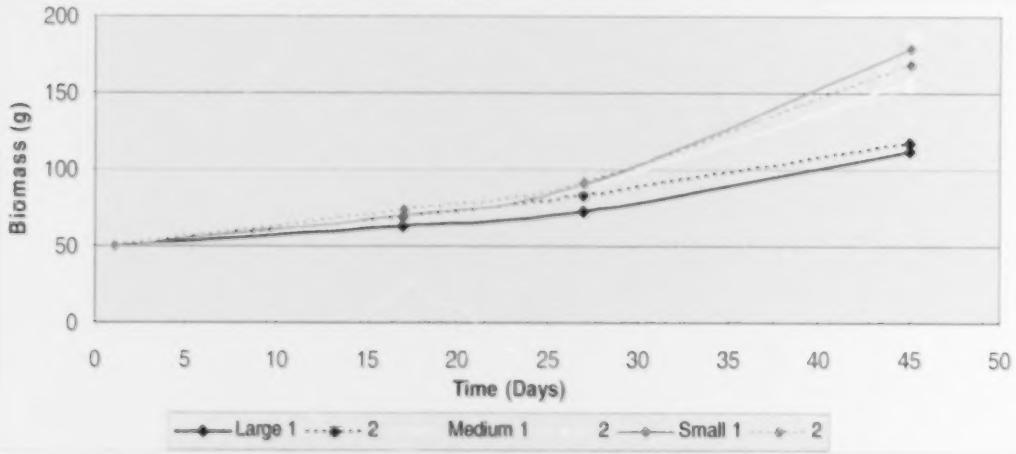


Figure 11. Oyster spat growth at different grades (small, medium, large). Sample size is 50.

A. Length and width increase +/- SD of the three grades over 45 days.



B. Biomass increase of the three grades over 45 days.



C. Percentage increase in length, width and biomass over the last 20 days

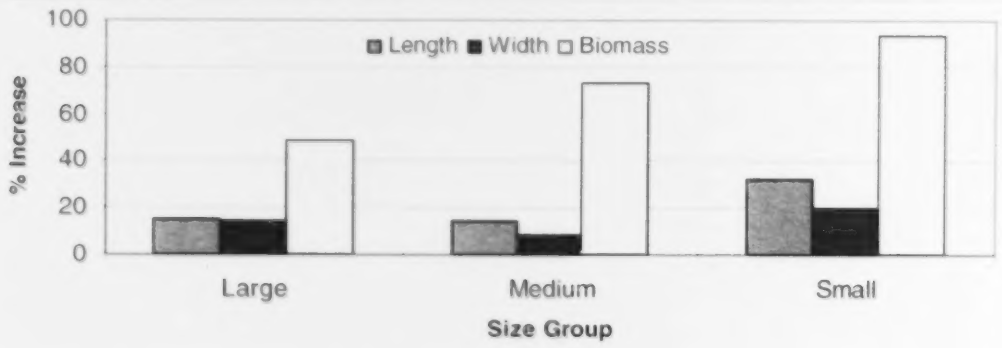
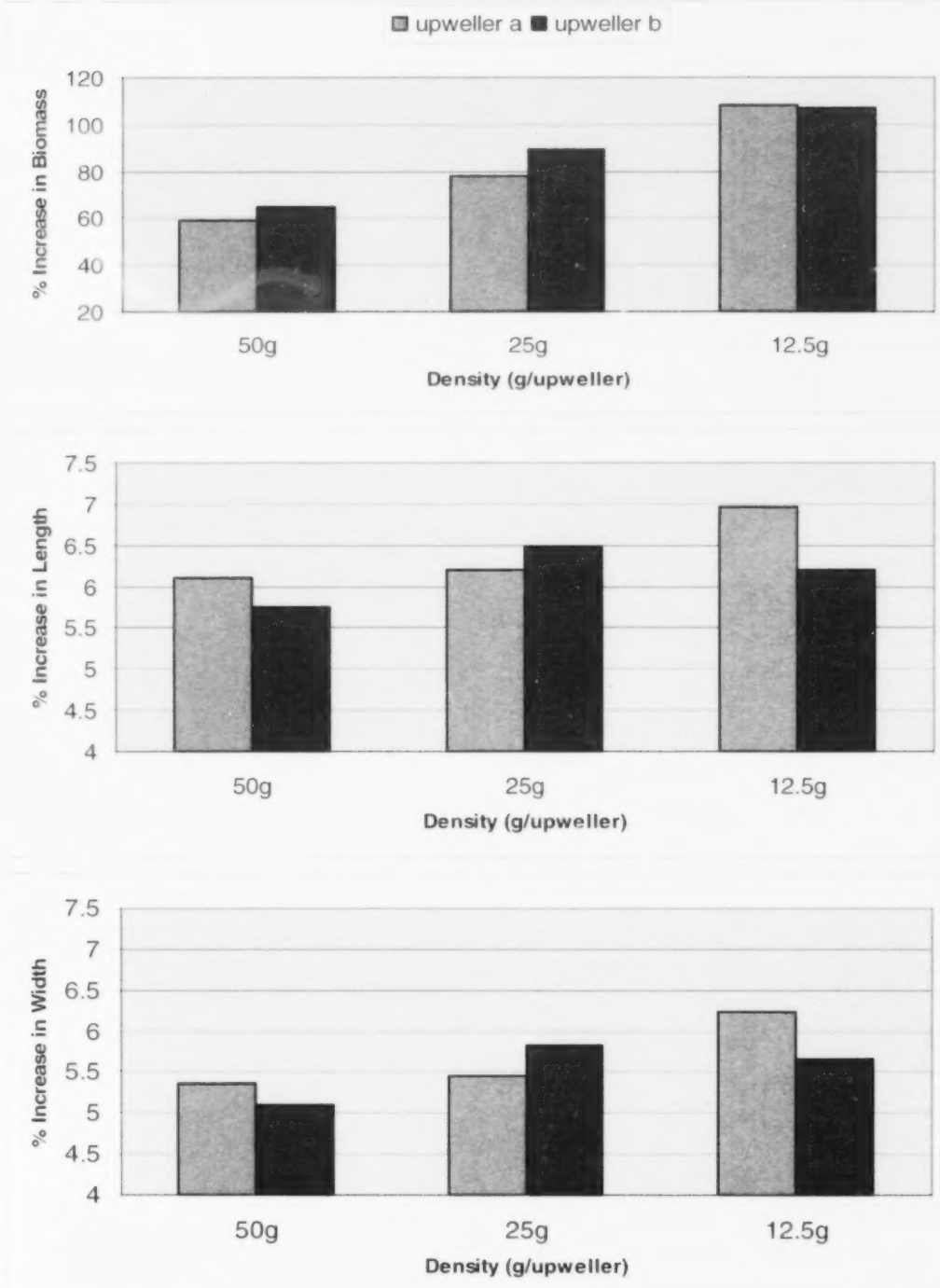


Figure 12. Percentage increase in biomass, length and width of the small oyster group at three different stocking densities over 15 days.



Winter 2004

A third attempt was initiated on January 19, 2004 for a 6 week conditioning period with oysters from Port Medway (PM), Blind Bay (BB), Cape sable (CS), Sambro, NS and Lake Lockhart, NB. The number of BHUs doubled compared to the previous year and this set-up #3 also included group spawns of 3-4 individuals to improve the chances of obtaining some larval releases. The different units involved controlled crosses between suspected inbred individuals, out-breeding crosses between hatchery and re-naturalized populations, and group spawns within populations.

Set-up #3

CS x CS	3 L Lockhart	3 L Lockhart	CS x **CS**	**CS x CS**	CS x CS
4 Blind Bay	**2 LL**	3 L Lockhart	**PM x BB**	**PM x BB**	CS x CS
PM x PM	PM x PM	4 Blind Bay	CS x BB (1)	**CS x BB**	PM x BB
CS x **CS**	**CS x CS**	**3 PM**	CS x BB (2)	CS x BB	**CS x BB**
CS x CS	**PM x PM**	**PM x PM**	**3 Sambro**	**4 Sambro**	**CS** x BB

Total: 71 oysters Successful releases in red Mortalities in blue
Units continued in next set-up in bold

The results of the controlled matings are as follows:

Out breeding crosses:

- PM x BB: release on March 2, set at D17-20, spat died on D43, likely from a bacterial infection (spirillum or spirochetes, see Figure 13)
- CS x BB (1): release on March 21, died on D15
- CS x BB (2): release on March 28, set and died on D15-17 (spirochete infection, Figure 14)

Related cross:

- CS x CS: release on March 19, died on D7

Group spawns:

- 4 Blind Bay: release #1 on March 3, died at D14 and release #2 on March 17 died on D9, both with velum deformities (Figure 13). Genotyping of larvae at 6 loci confirmed that this spawn was the result of a mating between 2 out the 4 individuals
- 3 Lake Lockhart: release #1 on March 10, set on D14-19, died on D28 from unknown reasons and release #2 on March 10, died on D14-19 (good velum but not feeding/swimming properly). This spawn is the result of mating of 2 out of the 3 individuals

The spirillum/spirochetes infection may have originated from a contaminated algal culture but was undetected in our testing.

Figure 13. Possible spirochete infection in spat cultures

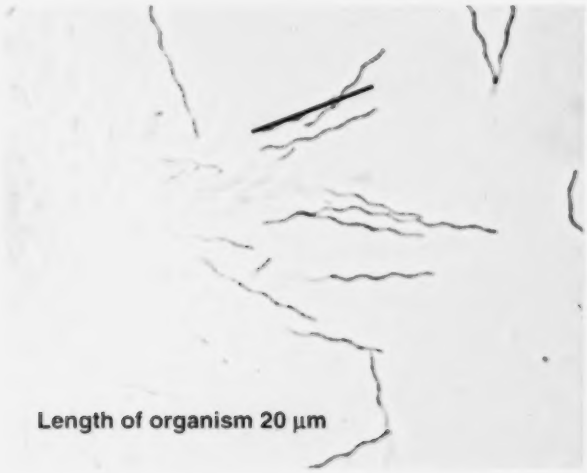
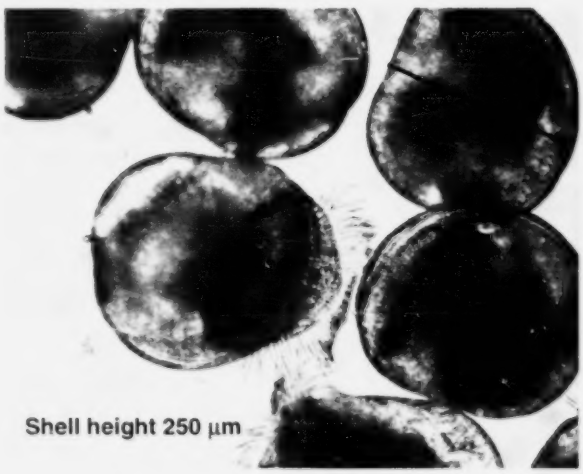


Figure 14. Larval velum deformities



Spring 2004

Set-up #3 continued (scaled-down) for an additional 6 weeks and the results are indicated below:

Set-up #3 continued with 15 units only

CS x CS	**PM x BB**	**PM x BB**
4 Blind Bay	LL X LL	**CS x BB**
PM x PM	**CS x BB**	4 Sambro
3 Sambro	**CS x CS**	**3 PM**
CS x CS	**PM x PM**	**PM x PM**

Successful releases in red

Related cross:

- LL x LL, release on April 17, set on D23-27, spat died at D58

Group spawns:

- 4 Sambro: release on April 10, died on D14
- 4 Blind Bay: release on April 11, died on D17-D24 (2 different temperatures, treated with antibiotics and bleach dips)

4.3. Conclusions

Other researchers have found that success of pair mating was higher than the theoretical 50% (IFREMER, 1995). This may mean that there is a mechanism of sex orientation between partners during the conditioning period, or it may be the result of selfing within the pair. Overall, there is no indication that selfing occurred in the European oysters used in this study. Out of the 13 "single" units, none released larvae. In the units with two oysters, there is a 50% theoretical percentage of putting together a male-female pair; however, in:

- Set-up #1 (spring 2003), out of 8 "pair" units, 4 spawned and released larvae,
- Set-up #2 (summer 2003), out of 9 units: 4+ should have spawned , only 1 did (**successful cross Sambro x LL**), and,
- Set-up #3 (winter/spring 2004), out of 25 units (8 group spawns): >12+ units should have spawned, only 9 did release larvae (including 4 group spawns).

These results show a much lower percentage of success than the theoretical value of 50%, measured as larval release, and this indicates that conditioning, gamete quality and environmental factors may have contributed to the lower success, especially in the context of the subsequent mass mortalities of larvae and spat. Nonetheless, **the husbandry (see Appendix B) of the successful crosses did not differ from the unsuccessful crosses.** The estimation of performance between the different groups could not be performed.

The full-sib spat from the only 2003 successful cross were transferred in July 2004 to grow-out sites in Lunenburg Bay, Tatamagouche Bay and Lobster Bay for assessment of survival rates in three different environmental conditions.

CONCLUSION AND FUTURE DIRECTIONS

The 2002/2003 ACRDP study and the present 2003/2004 ACRDP project provided the first analyses of the genetic structure of *Ostrea edulis* populations/stocks in Nova Scotia. They are based on variation at microsatellite loci, and demonstrate the utility of these markers for discerning population diversity and population structure. We are currently able to use a set of 6 markers that constitute an important tool to create pedigrees and breeding programs with limited inbreeding and high levels of genetic diversity. However, we could benefit from new loci development as some of the microsatellite loci reveal some limitations (*e.g.* null alleles, low number of alleles).

While the impact of inbreeding and heterosis on performance (survival, growth, settlement success) could not be evaluated in this project, we found no evidence of selfing (self-fertilization) but evidence that mass spawning is dominated by a few individuals that spawn in factorial crosses. Also, a procedure was developed to extract DNA and genotype larvae as small as 180 µm (D2).

Current hatchery production for aquaculture allows for the development of genetically improved strains. Simultaneously, this may lead to loss of genetic diversity and excessive inbreeding, which can have adverse effects. High levels of inbreeding can lead to an overall decline in fitness known as inbreeding depression (Backus et al., 1995). The possible manifestations of inbreeding depression include reduced survival and growth rate, loss of reproductive performance, and increased susceptibility to epidemics due to loss of genetic diversity. For example, Mallet and Haley (1983) and Naciri-Graven et al. (2000) have observed that in oysters, growth performance of offspring is negatively correlated with the relatedness of their parents.

The impact of grading spat has been evaluated in this project as well. Smaller oysters (typically believed to have limited potential for growth) showed increased growth rate (% mass and size) when competition from larger oysters was removed and when density was lower. Grading too harshly is similar to selecting for fast growing spat in the hatchery who are not necessarily fast growing adults at grow-out sites. Discarding small spat may not only genetically impoverish the next breeding stock but may also hinder selection for growth.

The BIO Shellfish research hatchery staff will continue supporting *Ostrea edulis* research and development by:

- providing genetic expertise for projects involving European oysters (e.g. recruitment success in Lake Lockhart (University of Moncton),
- providing a follow-up on the 2003 cross and remaining broodstock in Nova Scotia,
- collaborating with the Shellfish Health Unit in Moncton, NB for future disease tests,
- continuing controlled spawnings on a research scale following recommendations for broodstock genetic management (Gaffney *et al.*, 1992), such as maximizing the number of broodstock, introducing individuals from a regional naturalized population (e.g. Lake Lockhart) at regular intervals, pooling offspring from multiple spawning groups (i.e. multiple lots), performing reasonable grading, and keeping oyster cultures at low densities. Those measures should be undertaken in order to maintain genetic diversity and prevent further erosion of genetic diversity in hatchery-based populations, and,
- refining husbandry control, monitoring water quality in hatchery, experimenting with disinfectants/antibiotics and strengthening the IFREMER-DFO collaboration on hatchery issues.

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Appendix A. Population statistics and allele frequencies.

Legend: PAC: Pacific Coast, LUN: Lunenburg, MED: Port Medway, CAS: Cape Sable, BLB: Blind Bay, SAM: Sambro, LLO: Lake Lockhart, CUH: Cundy Harbor, BOH: Boothbay Harbor, BHB: Blue Hill Bay.

Population Statistics

Population	Pop size	Loci typed	Exp Hz	Obs Hz	# Alleles
PAC	30	5	0.860	0.827	11.20
LUN	68	5	0.810	0.670	11.40
MED	39	5	0.786	0.736	10.80
LLO	148	5	0.821	0.707	16.40
SAM	57	5	0.853	0.705	13.80
CUH	100	5	0.876	0.720	18.80
BOH	89	5	0.889	0.793	18.20
BHB	65	5	0.854	0.753	14.60
CAS	125	5	0.796	0.698	13.60
BLB	36	5	0.773	0.678	9.00

Appendix A cont'd. Population statistics and allele frequencies.

Allele frequencies for all populations by locus

Locus	Populations									
U2	PAC	LUN	MED	LLO	SAM	CUH	BOH	BHB	CAS	BLB
150						1.25				
154				0.43		5.00	1.14			
156	1.67			0.43		2.50	1.14	0.91		
158				1.29		2.50		3.64		
160	3.33	11.76	1.39	9.48	2.70	5.00	6.82	8.18	2.58	
162	15.00	18.38	2.78	7.33	6.76	8.75	5.68	1.82	16.49	23.08
164	1.67	0.74	1.39	5.17	6.76	8.75	4.55	4.55	0.52	
166	8.33		1.39	0.86		28.75	20.45	6.36	1.55	
168	3.33	7.35	1.39	3.88	10.81	2.50	4.55	1.82	7.22	4.62
170		10.29	6.94	12.07	1.35	1.25	2.27		1.55	3.08
172	8.33	8.09	26.39	6.47	6.76	2.50	10.23	10.91	8.76	6.15
174				0.86		1.25	1.14	5.45	0.52	
176	13.33				1.35	5.00	2.27			
178		2.94	5.56	7.33	13.51		3.41	5.45	10.31	12.31
180		1.47		8.19	5.41	3.75	5.68	21.82	8.76	
182	5.00	3.68	15.28	1.29	5.41	2.50	6.82	0.91	4.12	15.38
184	13.33	13.97	2.78	11.21	21.62	3.75	5.68	7.27	12.89	7.69
186	5.00			3.02		3.75	2.27			
188	10.00	3.68	5.56	1.72	1.35		4.55	1.82	2.58	
190				2.59	5.41	2.50		1.82	0.52	
192	1.67	9.56	6.94	7.33	9.46	2.50	4.55	10.91	7.22	10.77
194	3.33					1.25	2.27	0.91		
196	3.33			2.16				0.91		
198		6.62	19.44	2.16		3.75	1.14	1.82	10.31	12.31
200		1.47	1.39	2.16				1.82	2.58	1.54
202	3.33			1.29	1.35		2.27	0.91		
204							1.14			
206			1.39	1.29					1.55	3.08
214						1.25				

Appendix A cont'd. Population statistics and allele frequencies.

T5	PAC	LUN	MED	LLO	SAM	CUH	BOH	BHB	CAS	BLB
106	8.33	6.62	1.28	6.29	10.91	11.49	6.90	11.54	3.91	6.90
114		5.88	1.28	3.85		5.17	2.30	4.62	1.30	10.34
116						0.57				
118							1.15			
120	16.67	3.68	1.28	7.34	0.91	12.64	13.79	6.92	3.48	
122		5.88	3.85	4.55	8.18		0.57	2.31	11.30	10.34
126	6.67	15.44	24.36	9.79	2.73	20.11	13.79	20.77	20.87	17.24
128						0.57	4.02	2.31		
130	1.67	8.09		5.94	4.55	1.15	9.77	7.69	0.43	1.72
132					2.73	1.72	2.87		0.43	
134				1.75	5.45	0.57	3.45		0.43	
136	6.67	5.88	5.13	4.90	9.09	4.02	2.87	1.54	7.83	1.72
138		1.47	12.82	3.15	4.55	13.79	10.34	6.92	3.04	
140	3.33				3.64	1.72	2.30	0.77		
142	10.00	2.21	1.28	11.89	17.27	7.47	2.87	7.69	5.65	5.17
144	21.67	25.74	23.08	14.34	14.55	5.17	9.77	13.08	16.52	17.24
146	13.33	7.35	6.41	8.04	0.91	1.15	2.87	5.38	1.74	
148	5.00	11.76	16.67	12.94	10.91	2.30	0.57		20.87	20.69
150	1.67			1.75	0.91	2.30	1.15		0.43	3.45
154			1.28	1.40	1.82	2.87	2.30	6.15	0.87	3.45
156			1.28	1.40	0.91	1.15	1.15		0.87	1.72
158				0.35		1.15	1.15			
160				0.35				1.54		
162						1.72	1.15			
164						0.57	0.57			
166						0.57	1.72			
168	5.00									
174							0.57	0.77		

H15	PAC	LUN	MED	LLO	SAM	CUH	BOH	BHB	CAS	BLB
175	20.00	4.62	2.56	10.53	14.42	21.43	27.22	4.17	21.88	7.35
179	6.67			0.38		3.30	2.53			
183					4.81	0.55				
187	11.67	17.69	26.92	21.05	19.23	2.20	5.70	7.50	25.45	30.88
191		1.54	7.69	4.14	4.81	8.24	9.49	10.00	3.57	
195		0.77	2.56	1.13	5.77	5.49	3.16	1.67		1.47
199	1.67	1.54				1.10				
203	20.00	20.00	28.21	21.05	1.92	12.64	11.39	13.33	16.52	10.29
207				1.88	0.96	2.20	3.16			1.47
211	3.33					5.49	2.53			
215	3.33	11.54	5.13	7.14	13.46	10.99	13.92	25.00	5.80	5.88
219				3.01		4.40	1.27	5.83		
223	33.33	34.62	21.79	25.56	24.04	20.88	19.62	26.67	22.32	30.88
227		7.69	5.13	4.14	10.58	1.10		5.83	4.46	11.76

Appendix A cont'd. Population statistics and allele frequencies.

O9	PAC	LUN	MED	LLO	SAM	CUH	BOH	BHB	CAS	BLB
145	6.67	2.21	1.28	3.82	4.72				1.67	
147						0.52	0.59			
149						1.04				
153						1.04				
155						4.17	0.59			
157			1.28	1.39	0.94	1.56	2.35	0.77		4.29
159						4.69	7.65	13.85		
161	6.67	9.56	1.28	6.94	22.64	7.81	7.65	11.54	3.75	5.71
163	3.33		2.56	1.39	2.83	6.25	9.41	9.23	3.75	
165	10.00	3.68	5.13	1.74	8.49	8.85	8.82	3.08	1.25	1.43
167	31.67	27.21	37.18	28.47	16.04	25.52	23.53	23.85	22.92	27.14
169	25.00	13.24	24.36	26.39	13.21	18.75	20.00	24.62	24.17	32.86
171	6.67	9.56	11.54	10.07	11.32	10.94	10.59	5.38	14.58	15.71
173	3.33	31.62	15.38	17.01	15.09	6.77	4.71	4.62	19.58	12.86
175		1.47		1.39	2.83			1.54	0.42	
177	6.67	1.47		1.39		1.04	4.12	0.77	7.92	
179					0.94	0.52		0.77		
181					0.94					
183						0.52				

J12	PAC	LUN	MED	LLO	SAM	CUH	BOH	BHB	CAS	BLB
219	15.00	4.62	5.13	3.10		11.22	5.11	1.54	1.23	
225		2.31					1.14			
227	23.33	53.85	53.85	60.69	46.30	34.18	28.41	40.00	63.11	67.14
229	11.67	6.15	11.54	3.45	1.85	1.02	2.27	0.77	3.28	2.86
231	1.67			1.72	0.93	4.08	3.41	2.31	0.82	
233		8.46				1.02	2.27			
235	6.67	1.54	21.79	5.52	12.04	2.04	1.14	19.23	12.30	
237		2.31			0.93		1.14	0.77	1.23	
239	1.67	1.54	1.28		0.93	7.14	1.70	4.62	0.41	
241	3.33			0.34		1.53	4.55		0.41	
243	3.33	6.15	1.28	2.76	1.85	2.55	6.82	1.54		
245	6.67	5.38	1.28	3.10	3.70	5.61	7.39	5.38	0.41	5.71
247		2.31	1.28	2.76	20.37	8.67	2.84	8.46	9.43	12.86
249	20.00		2.56	3.10	2.78	1.02	2.84	5.38	3.28	
251		3.85				3.57	10.80	5.38		
253				2.41	0.93	3.06	4.55			1.43
255				1.72	3.70	5.10			0.41	
257	6.67			3.10	0.93	4.08	4.55	0.77		5.71
259				1.03		3.06	4.55	2.31	0.41	
261		1.54		3.79	1.85	0.51	2.27	1.54	2.46	4.29
263				0.34	0.93		0.57		0.41	
265						0.51	1.70			
267				0.69					0.41	
269				0.34						

Appendix B. Detailed protocols for changing European Oyster larvae and spat.

Change Schedule: Mondays, Wednesdays and Fridays (and Saturday evenings if larval health becomes an issue)

Figure 15. Downwellers placed in tanks

a) Advanced Preparation

1. Turn UV system on and let the water flow for approximately 5-10 minutes and ensure the system is functioning properly.
2. Prepare a diet from non-contaminated algae carboys while UV system is warming up.
3. Rinse out tanks using UV Filtered Sea Water (UVFSW) and commence filling the appropriate number of tanks needed for the change. Place an air stone in the tanks while filling to ensure the water is being off-gassed.
4. **(If necessary)** Place heater into the newly filled tank and heat to 20°C.
5. Take a small sample of larvae from the top of the tanks to be changed and examine them under a microscope to access larval health and development. Also, visual observations such as swimming behaviour and absence / presence of food in the tank should be noted.

b) Draining the Tanks

1. Open the tank valve for approximately 2 seconds and then close the valve. This allows stagnant water to escape.
2. Rinse previously cleaned screens, trays and elbows with UVFSW before use.
3. Take a PVC tank elbow and fit the elbow into the tank valve. Place a 125µm+ screen into a catch tray and then place the screen/tray on the floor underneath the elbow.
4. Slowly, open the tank valve. Adjust the flow so that the water does not flow over the sides of the screen. Drain the tank onto the screen until there is just enough water left in the tank to cover the bottom. Then close the tank valve.
5. Take the screen out of the catch tray, rinse the larvae thoroughly with UVFSW on the screen, then invert it over a clean, filled, heated tank and rinse the larvae into the tank or

into a cleaned graduated bucket for concentrating larvae for sampling (counting). Repeat rinsing until all of the larvae are rinsed off of the screen.

6. Remove the elbow from the valve of the tank and place the same 125µm+ screen back on the floor under the valve. Open the tank valve and gently tilt the tank forward, allowing the water at the bottom to drain. Next, use the UVFSW to rinse the larvae off of the sides and the bottom of the tank. Repeat rinsing until all of the larvae are rinsed onto the screen. Rinse all remaining larvae from the screen into the new tank or sampling bucket. If grading is required, it should be performed at this step.
   Note: If there is any debris on the screen amongst the larvae, it can be removed by rinsing the larvae through a larger screen (250µm+) over top of the tank, prior to placing them back into the tank.

c) Setting Animals

1. As larvae approach 300µm+ in size they will begin to set, so larvae need to be inspected under microscope everyday thereafter to look for eyespots and foot activity.
2. Once the majority of the larvae have eyespots and foot activity, a PVC disk coated with Extolité (a setting solution provided by André Mallet, MRS) is lowered onto the bottom of the tank so the animals will set on the PVC rather than the tank itself.
3. On the next change day, or sooner, depending on how heavy the set is, change the tank according to **Procedure b**. Leave a few inches of water in the bottom of the tank and lightly spray off the PVC disk to get any remaining larvae off the PVC. Pull the disk out of the tank and set it aside and finish rinsing the larvae out of the tank and place the larvae in their new tank with a new PVC disk.
4. Fill the bottom of the tank that was just drained with 50mm of UVFSW and place the PVC disk back in the tank. Resting the disk at a 45^0 angle against the bottom of the tank and the top wall of the tank, use a clean, sharp razor blade to scrape the spat off of the PVC disk. Scraping in long, uniform motions with UVFSW flowing over top of the disk and razor blade, scrape the entire disk (front and back) into the tank.
5. Rinse the tank down using UVFSW and collect the spat on a screen the same way the larvae were collected and place them in a downweller system. Downweller mesh size will range from 200µm+, depending on spat size and age.

d) Changing Spat Tanks (Downwellers)

1. Fill tanks according to **Procedure a**.
2. Slowly open the valve on the bottom of the tank with the downwellers and let the water drain out.
3. Using the UVFSW, lightly apply pressure to the end of the hose and spray off the downwellers, being careful not to spray any oysters out of the downwellers. Unhook any air lines connected to the downweller.
4. Pick up the downwellers and transfer them to their new tank. Be sure that the volume of water in the tank is not too high so that the spat can float out into the main tank.
5. Give each downweller a quick spray with domestic water to break any surface tension allowing the oysters that are floating on the surface to sink to the bottom of the downweller. Using a small transfer pipette, rearrange and spread the oysters out evenly on the bottom of the downweller.
6. Reconnect the air line to each downweller and make sure it is functioning properly. Downwellers should be washed weekly or whenever they appear dirty.
7. Once oysters have reached 2mm+ in size, they can be transferred into upwellers.

e) Washing Tanks and Equipment

1. Thoroughly rinse the lid, the inside of the tank, and around the top of the tank with domestic water.
2. Wash tanks (12% commercial bleach solution is mixed in 20L bucket with reversed osmosis water and 5ml/L of bleach). Pour 1-2 litres of the bleach solution into the dirty tank. Using a cloth, scrub the lid, the inside of the tank, and around the top of the tank.
3. Using a brush, clean the valve thoroughly.
4. Wait 5 minutes and repeat step 2 and 3.
5. Rinse the tank and the lid thoroughly 5-7 times with domestic water.
6. Empty all the water out of the tank and replace the lid for storage until the next change day or rinse with UVFSW, fill and heat for immediate use.
7. Wash remaining equipment (elbows, trays, buckets, etc...) with the bleach solution (see #2 above). Soak screens in the bleach solution for 5 min. Rinse well with domestic water and allow to dry.

f) Algal cultures and diets

1. Phytoplankton is grown in 10L polycarbonate containers (carboys), 1 μm filtered UV treated enriched seawater and autoclaved.
2. The carboys are inoculated with phytoplankton cultures and placed on shelfs in front of cool white/grow fluorescent light bulbs, in a 200C temperature controlled room.
3. Samples from the carboys are aseptically transferred onto prepared marine agar plates 1 to 3 days prior to harvest. Plates are incubated at 20oC
4. The agar plates are checked for bacterial growth prior to the harvesting of the carboy. If any growth is detected on the agar plate, the corresponding carboy is discarded.
5. Diets are prepared with uncontaminated carboys and may be altered from the preferred composition by avaibility. Generally, the young larvae receive 30-35,000 cells/mL and spat >50,000 cells/mL
6. Composition of the diets also changes as the larvae grow, from smaller cells TISO/MONO/CCal to the larger CHGRA/Actin/Tet. TISO and MONO generally remain part of the diet throughout and PAV 459 is also included when available.
7. Diets are added to the tanks after they have been cleaned, filled, heated and larvae returned. Additional feed is added as required.

Appendix C. Protocol for genotyping European Oyster larvae

Larvae were placed in a Petri dish filled with water and individuals were isolated and selected using a 10µL pipette and a fine gauge needle. Individual larvae were placed in separate wells of a 96-well PCR plate with 90µL of Qiagen ATL lysis buffer. Larvae were crushed using a needle and forceps. 10µL of proteinase K was added and the larvae were digested at 55oC. The rest of the extraction was carried using Qiagen DNeasy tissue kit, following manufacturer's instructions (Qiagen cat. # 69506), except in the final elution step, where DNA was eluted in only 20µL of AE buffer rather than 200µL. The rest of the genotyping protocol can be found in Vercaemer *et al.*, 2003.

Table 7. Resolution of genotyping *Ostrea edulis* larvae for 5 loci

Locus	% larvae successfully genotyped out of 18	Earliest date for successful genotyping
H15	89%	D2
O9	94%	D2
J12	61%	D14
T5	56%	D14
U2	22%	D48